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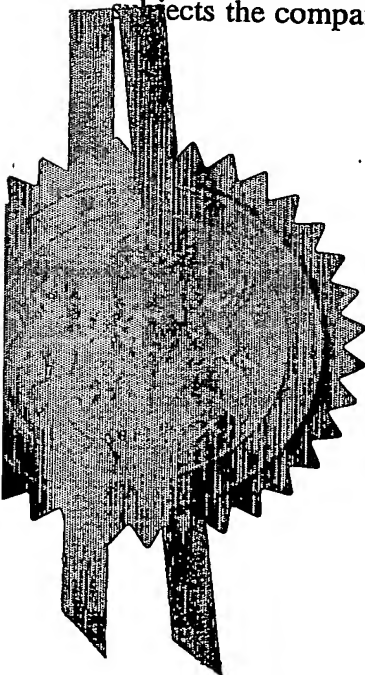
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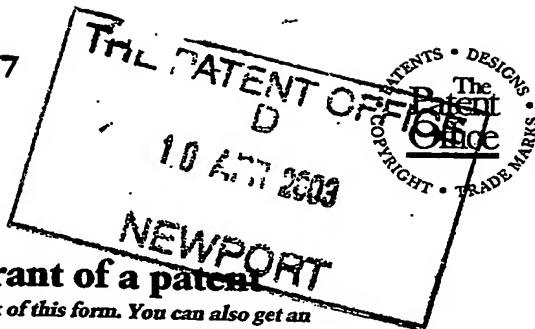


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10APR03 E799217-1 D01559  
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3. Full name, address and postcode of the or of each applicant (underline all surnames)

University of Wales College of Medicine  
Heath Park  
Cardiff  
CF14 4XN

Patents ADP number (if you know it)

798546001

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

PROTEOLYSIS METHODOLOGY

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Wynne-Jones Laine & James  
Morgan Arcade Chambers  
33 St Mary Street  
Cardiff  
CF10 1AF

Patents ADP number (if you know it)

1702002- 8585531001

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Country	Priority application number (if you know it)	Date of filing (day / month / year)
GB	0226441.4	12.11.02

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Number of earlier application	Date of filing (day / month / year)

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Claim(s)

03

Abstract

01

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Signature

*Wynne-Jones, Laine & James*  
Wynne-Jones Laine & James

Date 09 Apr 2003

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## PROTEOLYSIS METHODOLOGY

The invention relates to a novel method for determining the significance of polymorphisms or mutations in a protein.

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Since the advent of gene sequencing technology in the late 1980's and the establishment of the human genome project in 1990 an enormous amount of information has been discovered about the sequence, or nature, of each gene in the human genome. Moreover, as the human genome project has developed the methods used to sequence genes have evolved considerably and this had led to the detection of variations within genes. Given that a typical gene could be 30 kilobases in length and that variations occur on average every 1100 bases, it follows that a tremendous amount of work needs to be undertaken in order to determine which variants are of clinical or technological significance.

15

However, this is a prerequisite step if one is to exploit the knowledge available in the human genome project and so be in a position to understand, for example, the human condition, and particularly human diseases, and factors that may influence same and so lead to new therapies.

20

Typically, investigators in the field of human genetics who have obtained the sequence of the normal, or wild-type gene, set about looking for significant changes in the gene by sequencing nucleic acid molecules from individuals who are thought to harbour a gene variant. Such individuals are people showing the symptoms of a specific disease which is thought to be related to the dysfunction

of a particular gene. Once a gene variant has been sequenced and compared with the wild-type further investigations are then undertaken to examine the cell biology of the protein encoded by the variant gene. The results of these investigations are then examined in the light of the physical symptoms in order to deduce a correlation.

It therefore follows that unravelling the nature of a gene variant and relating it to function and then clinical symptoms is a long and tedious process, especially when one considers that a given gene can be 3.6% polymorphic. It is therefore apparent that simply identifying which variant to investigate further can be a difficult step in itself. This is true not only for the field of human genetics but also in respect of studies of other animal and plant species.

With this in mind, we have developed a novel assay for quickly and efficiently determining the likely significance of a gene variant.

Our novel methodology is based upon the basic structure of proteins.

The basic structural unit of a protein is an amino acid. An amino acid consists of an amino group, a carboxyl group, a hydrogen atom and a distinctive R group bonded to a carbon atom, conventionally known as the side chain. There are 22 amino acids and any number and combination of them are able to join, via peptide bonds, to form a sequence or chain of amino acids known as peptides. Thus the sequence of bonds running the length of the peptide chain is known as

the backbone. Additionally, bonds may also exist between side chains of amino acids as a result of the formation of disulphide bonds thus forming crosslinks between separate peptide chains. Adjacent peptide chains can therefore join to form a secondary structure such as dimers or trimers etc. The secondary structures can then fold, due to the nature of the interaction of adjacent amino acids, to form a three dimensional tertiary structure. This tertiary structure represents the active form of the protein and may comprise sites, or pockets, into which other molecules fit in order to activate the protein or allow the protein to respond thereto.

Digestion, or break down, of proteins in a controlled fashion, occurs all the time during the process of digestion. A class of enzymes known as proteases perform this function. They basically attack specific bonds in order to cleave the protein at sites where these bonds exist. It follows that different proteins will have different susceptibilities to various enzymes depending upon their primary structure.

Whilst all this information is known, no-one has thought to take advantage of it before in relation to genetics and, in particular, using it as the basis to tackle the large number of genetic variants that exist in order to determine which are the clinically, or technologically, significant variants.

Accordingly, we have used this information to develop a novel assay which can screen any number of variants, simultaneously if required, in order to determine

which, if any, require further investigations.

Our methodology is quick, efficient and inexpensive to perform.

5     Statements of Invention

According to the invention there is therefore provided a method for determining the effect of a given nucleic acid polymorphism or mutation, in a nucleic acid molecule, on the structural properties of a protein encoded by said nucleic acid molecule comprising:

- 10     a)     exposing the protein encoded by said nucleic acid molecule to at least one protease; and
- b)     determining whether or to what extent proteolytic cleavage takes place; and, optionally,
- c)     comparing this proteolytic cleavage with that of the wild-type protein when
- 15            exposed to the same protease(s).

In a preferred embodiment of the invention said protein encoded by said nucleic acid molecule or gene variant is exposed to a plurality of proteases and ideally different proteases which attack different bonds. Proteases that are suitable for

20     use in the methodology of the invention include: Trypsin, chymotrypsin, proteinase K, aminopeptidase, carboxypeptidase, collagenase, elastase, Kallikrein, metalloendopeptidase, papain, pepsin, and indeed any other known protease.

Notably, where cleavage is different from that exhibited by the wild-type, one would conclude that the variant, or indeed a combination of variants, was significant. This is because the variant(s) would either render the protein more vulnerable to digestion or confer resistance to digestion as a result of alteration(s) to the tertiary, or structural, form of the protein.

In yet a further preferred embodiment of the invention a plurality of proteins encoded by a plurality of genetic variants are tested in parallel and thus the methodology of the invention may be performed as a screening methodology where a plurality of incubation receptacles are filled with a plurality of proteins to be tested and then said proteins are exposed to a selected protease, or group of proteases, either simultaneously or successively, or vice versa.

More preferably still, the methodology of the invention involves incubating the protein(s) to be tested with the said protease(s) under conditions that support the activity of the relevant enzyme(s). For example, this may involve exposing the test protein to the enzyme at a temperature at which the enzyme is optimally functional, such as 37°C, and for a time sufficient for the enzyme to perform its activity, for example between 15 minutes and 1.5 hours.

More preferably still, after a suitable length of time the incubation period is terminated, for example, by adding an enzyme inhibitor to the incubation receptacle. Finally, proteolytic cleavage is assessed using any conventional protein assay technique such as, for example, SDS-PAGE analysis either



followed by staining the gel (coomassie blue or silver staining) or by western blotting. Optionally, additional studies may then be undertaken to determine the functionality of the protein variant.

5 In yet a further preferred embodiment of the invention the technique undertaken, in order to determine the extent of proteolytic cleavage, involves assaying not only each test protein but also the wild-type protein that, ideally, has been exposed to the relevant enzyme(s) and a sample of the wild-type and test protein(s) that has not been exposed to the relevant enzyme(s). In this way, a  
10 positive and a negative control are included in the assay for the purpose of determining the amount of proteolytic cleavage that the test protein exhibits vis a vis the wild-type protein and also the background level of protein degradation experienced as a result of the assay conditions.

15 It is to be understood that the invention is not to be limited to the specific assay that is chosen to assess proteolytic cleavage, rather the invention, principally, lies in the use of the technique of proteolytic cleavage to assay genetic variants.

20 It follows from the information above regarding the tertiary structure of the protein that the nature of the amino acids in the peptide chain will determine the protein folding and so susceptibility to different enzymes. In turn, the nature of the amino acids in the peptide chain will be determined by the nucleic acid coding sequence and so variations in this sequence will lead to variations at the amino acid level and so differential protein folding and thus variable

susceptibility to proteolytic cleavage.

Given that the assay is quick and efficient to perform, a whole range of proteins, each coded by a genetic variant for a given gene, or more than one gene, can be simultaneously assayed in order to determine which variant gives rise to a change in the tertiary structure of the amino acid and thus which is most likely to affect the functioning of the protein.

An embodiment of the invention will now be described by way of example only, with reference to variants in the growth hormone gene (*GH1*) and the following examples.

### **Experimental Subjects**

A total of 74 pre-pubertal Spanish children exhibiting short stature were referred from a number of paediatric endocrine clinics in Andalusia. These children adhered to the definition of familial short stature proposed by Ranke (1). The height standard deviation score (SDS) of all the children in the study was -2 SDS below the mean for the general population. All subjects exhibited normal GH secretion after a pharmacological stimulation test (peak GH values  $\geq 10$  ng/mL). Pharmacological tests used were clonidine (34 cases), propranolol (25 cases) and insulin (15 cases). Ethical approval for genetic studies was obtained from each participating centre and the Multi-Regional Ethics Committee. Written informed consent was obtained from each participating individual.

Standard deviation scores were calculated for height, body mass index, paternal and maternal heights, mid-parental height, IGF-1 and IGFBP-3 levels, peak GH secretion in ng/mL, and GHBP (as a percentage). These data are presented in Table 1 for the two patients (B4 and B49) in whom a novel *GH1* gene lesion was found and the cohort of patients studied.

## Materials & Methods

### *Polymerase chain reaction (PCR) amplification of a GH1-specific fragment*

Genomic DNA was extracted from patient lymphocytes by standard procedures. PCR amplification of a 3.2 kb *GH1*-specific fragment was performed as described (2).

### *Cloning and sequencing of GH1 gene-specific PCR fragments*

GH1 gene-specific (3.2 kb) PCR fragments were sequenced directly with BigDye v3.0 (Applied Biosystems, Foster City, CA) and analysed on an ABI 3100 DNA sequencer (Applied Biosystems) as described (2). Additional primers used for sequencing in the reverse direction were GHBFR (5' TGGGTGCCCTCTGGCC 3'; -262 to -278), GHSEQ1R (5' AGATTGGCCAAATACTGG 3'; +215 to +198), GHSEQ2R (5' GGAATAGACTCTGAGAAAC 3'; +785 to +767), GHSEQ3R (5' TCCCTTTCTCATTCATTC 3'; +1281 to +1264), GHSEQ4R (5' CCCGAATAGACCCCGC 3'; +1745 to +1730) [Numbering relative to the transcriptional initiation site at +1; GenBank Accession No. J03071]. Samples containing sequence variants were cloned into pGEM-T (Promega, Madison WI) followed by sequencing of a minimum of four clones per individual.

*In vitro expression and assay of biological activity of GH variants*

A cloned wild-type *GH1* cDNA incorporating a His tag on the carboxy terminal was modified using site-directed mutagenesis as previously described (3) to generate the Ile179Met Ile= isoleucine, Met= methionine variant.

This vector was then transfected into High Five insect cells (Invitrogen) as previously described (3), and human GH in the culture supernatants quantified by ELISA (DRG Diagnostics, Marburg, Germany). The cross-reactivity in the ELISA of the Ile179Met GH variant and insect cell-expressed wild-type GH was confirmed by dilutional analysis to be equal to that of the assay reference preparation (calibrated against the MRC 1<sup>st</sup> IRP 80/505 reference preparation). For Western blotting studies of the activation of the MAPK pathway, the His-tagged forms of wild-type GH and the Ile179Met variant were harvested and purified on nickel columns (Invitrogen) according to the manufacturer's instructions. GH was eluted from the column with 350mM imidazole. A buffer change step was carried out at this stage by concentrating the GH from the imidazole eluate by ultrafiltration (Centriplus YM-10, Millipore, Bedford, MA), washing in PBS and finally resuspending in PBS. The identity and purity (>95%) of the purified His-tagged GH was confirmed by SDS-PAGE with silver staining, and western blotting. The purified forms of GH were quantified as described above.

HK293 cells transfected with the full-length human GH receptor (GHR), and selected on the basis of elevated GHR expression (HK293hi cells), were used to assay the biological activity of the Ile179Met GH variant (4, 5). Cells were plated into 24-well plates (100,000 cells per well) for 24 hrs in DMEM:F-12 (1:1) containing 10% FCS. Cells were co-transfected overnight using a lipid-based transfection reagent according to the manufacturer's instructions (FuGENE 6, Roche Molecular Biochemicals) with a STAT 5-responsive luciferase reporter gene construct (4) and a constitutively expressed  $\beta$ -galactosidase expression vector (pCH110; Amersham Pharmacia Biotech) to correct for transfection efficiency. Cells were then incubated with variant and wild-type GH diluted to a known standard range of concentrations (0.1-10nM) in serum-free DMEM:F-12 (1:1) containing  $2.5 \times 10^{-7}$  M dexamethasone for 6 hrs to allow GHR dimerization, STAT 5 activation and luciferase expression. After incubation, cells were lysed and the luciferase measured in a microplate luminometer (Applied Biosystems) using the Promega luciferase assay system. Luciferase expression thus provided a measure of the degree of GHR activation and hence the biological activity of the GH variant. Experiments were carried out in quadruplicate and repeated at least 3 times. Statistical analysis of luciferase assay data was carried out by analysis of variance (ANOVA) with subsequent comparisons using the Student-Newman-Keuls multiple comparison test.

#### **GH secretion studies in mammalian cells**

Rat pituitary (GC) cells were transfected with a pGEM-T plasmid containing a 3.2 kb gene fragment spanning the entire wild-type *GH1* gene or the equivalent

construct for the Ile179Met variant. Cells were plated into 24-well plates (200,000 cells per well) and cultured overnight in DMEM containing 15% horse serum and 2.5% fetal calf serum (complete medium). The cells were co-transfected with 500ng *GH1* plasmid and  $\beta$ -galactosidase expression vector (pCH110; Amersham Pharmacia Biotech) to correct for transfection efficiency using the lipid-based transfection reagent Tfx-20 (Promega). Transfection was carried out in 200 $\mu$ l serum-free medium containing 1 $\mu$ l Tfx-20/well for 1 hr, after which 0.5ml complete medium was added to each well. Cells were cultured for 48 hrs, medium harvested and cells lysed for  $\beta$ -galactosidase assay to correct for differences in transfection efficiency. GH in the medium was quantified for all variants using a human GH ELISA (DRG Diagnostics) that showed no cross-reactivity with rat GH. Experiments were performed and data analysed as described for the biological activity assay.

#### *Proteolytic digestion of the GH variant*

Trypsin, chymotrypsin, or proteinase K (all Sigma, Poole, UK) were added to a final concentration of 0.1 $\mu$ g/ml to 100 $\mu$ l culture medium harvested from insect cells expressing either wild-type GH or the Ile179Met variant (60nM) and then incubated at 37°C for 1 hr. Previous dose-dependent studies on wild-type GH had shown that 0.1 $\mu$ g/ml was the lowest concentration at which GH degradation was detectable by all three enzymes. After the 1 hr treatment period, 10 $\mu$ l trypsin-chymotrypsin inhibitor (500 $\mu$ g/ml) was added to stop the trypsin and chymotrypsin digests and 1 $\mu$ l PMSF (0.1M) was added to stop the proteinase K digest. Each

reaction was then incubated for a further 15 mins at 37°C. Samples were analysed by SDS-PAGE on a 12% gel using a mini gel apparatus (Bio-Rad Laboratories, Hercules, CA). Equivalent amounts of undigested wild-type GH and Ile179Met variant that had been incubated for 1 hour at 37°C were also run on the gel. The gel was electroblotted onto PVDF membrane as previously described (6), probed with a mouse monoclonal anti-human GH antibody (Lab Vision, Fremont, CA), diluted 1:500, detected using an anti-mouse IgG-horse radish peroxidaseHRP (HRP) conjugate (1:5000, Amersham Biosciences) and visualised by enhanced chemiluminescence (ECL Plus, Amersham Biosciences). Films were analysed using the Alpha Imager 1200 digital imaging system (Alpha Innotech Corp, San Leandro, CA) and the results expressed as the amount of GH remaining following enzyme digestion as a percentage of undigested GH. The experiments were repeated 3 times and assessed statistically by a two-tailed t-test.

#### *Activation of the MAPK pathway*

The ability of the Ile179Met variant to activate the MAPK signal transduction pathway relative to wild-type GH was investigated by stimulating murine 3T3-F442A preadipocytes (7) with wild-type GH and the Ile179Met variant (20nM for 15 mins). Cells were then lysed and analysed by SDS-PAGE on a 10% gel as described above. The gel was blotted onto PVDF membrane and probed using monoclonal antibodies that detect the activated forms of p42/p44 MAPK phosphorylated on residues Thr202/Tyr204 (Cell Signaling Technology, Beverly, MA) and STAT 5 phosphorylated on residues Tyr694/Tyr699 (Upstate

Biotechnology, Lake Placid, NY). Western blots were processed, visualised using ECL Plus and the images analysed as described above. To ensure equal protein loading between lanes, blots were stripped and reprobed with rabbit antibodies that recognise total MAPK or STAT 5 (Santa Cruz Biotechnology, Santa Cruz, CA) as appropriate. Second antibodies were either anti-mouse or anti-rabbit IgG-HRP conjugates depending on the primary antibody used (1:5000, Amersham Biosciences).

#### *Molecular modelling*

The Ile179Met variant was structurally analysed by inspection of the appropriate amino acid residue in the X-ray crystallographic structure of human GH (PDB: 3HHR) [8]. The wild-type and mutant GH structures were compared with respect to electrostatic interactions, hydrogen bonding, hydrophobic interactions and surface exposure. Molecular graphics were performed using the ICM molecular modelling software suite (Molsoft LLC, San Diego, CA).

#### *Functional characterization of the Ile179Met variant*

The evolutionary conservation of the hydrophobic residue Ile179 was examined by ClustalW multiple sequence alignment of orthologous GH proteins from 19 vertebrates (9). This residue is a hydrophobic valine in all vertebrates except turtle, indicating that the substitution by Ile in the human lineage is conservative. Comparison with the paralogous genes of the human GH cluster revealed that the residue analogous to Ile179 is Met in CSH1, CSH2 and the CSH pseudogene



(CSHP1). This is consistent with the conservative Ile179Met substitution having been templated by gene conversion.

5 The Ile179Met substitution was then modelled by replacement of the residue in the X-ray crystallographic structure of human GH. Ile179 lies in helix 4 where it is partially exposed, allowing hydrophobic interactions with the side-chain of the "hotspot" GHR residue Trp169 (10, 11). Further interactions with the GHR occur between the side-chain and backbone atoms of Ile179 and the backbone atoms of GHR residues Lys167 and Gly168. Replacement of the Ile179 side-chain with  
10 the side-chain of methionine indicates that these hydrophobic interactions may be conserved upon substitution.

15 The Ile179Met variant was expressed in insect cells and a STAT 5 responsive luciferase reporter gene assay system (4, 5) used to assay its signal transduction activity. For GH to be biologically active, it must bind to two GHR molecules thereby triggering receptor dimerization and activation of the intracellular tyrosine kinase JAK2, which in turn activates the transcription factor STAT 5 by phosphorylation. Phospho-STAT 5 dimerizes, translocates to the nucleus and binds to STAT 5-responsive promoters thereby switching on the expression of  
20 GH-responsive genes. The assay of GH biological activity used here requires all stages of this pathway to be functional. The Ile179Met variant was found to display normal ( $99 \pm 4\%$  wild-type) ability to activate the JAK/STAT signal transduction pathway when compared to wild-type GH at a concentration of 1nM, the approximate  $ED_{50}$  value for GH in this assay system. It is possible that this

variant either exerts its deleterious effects on a signal transduction pathway other than JAK/STAT or its detrimental effects may not be manifest in a static *in vitro* system. Alternatively, the Ile179Met substitution could compromise GH folding, secretion or stability *in vivo*, or have adverse effects on the GH axis that are as yet undefined.

In order to investigate the activation of the MAP kinase pathway by the Ile179Met variant studies designed to assess the ability of the GH variant to activate this pathway were undertaken. These showed a significantly reduced level of activation in response to the variant ( $3.77 \pm 0.45$  times basal level of activation) compared to wild-type ( $7.31 \pm 0.7$  times basal level of activation; mean  $\pm$  SEM,  $n = 4$ ,  $p < 0.01$ ). This contrasted with its ability to activate STAT 5 to the same level as wild-type GH (40.3 times for the wild-type versus 42.7 times for the Ile179Met variant). The STAT 5 data confirmed the result from the STAT 5-responsive luciferase reporter gene bioassay showing the same level of activity between wild-type GH and the Ile179Met variant.

To explore these possibilities further, the secretion of the Ile179Met variant was studied in rat pituitary GC cells. The wild-type *GH1* gene, under the control of *GH1* promoter haplotype 1, was transfected into GC cells and shown to be responsible for the secretion of human GH (as measured using a human GH-specific ELISA) at a concentration of 64pM over a 48hr period. The level of secretion of the Ile179Met variant (also under the control of *GH1* promoter haplotype 1 with which it is associated *in cis* in patient B49) was measured and

expressed as a percentage of wild-type. Since secretion was found to be  $97 \pm 4\%$  of the wild-type value, it may be inferred that this mutation is likely to have little or no effect on GH secretion.

5 Finally, the Ile179Met variant was also challenged with trypsin, chymotrypsin and proteinase K to determine if it was more susceptible to proteolytic cleavage than wild-type GH. However, the 179Met variant proved similarly resistant to proteolytic cleavage as wild-type GH indicating that there were no significant differences in protein folding between the two forms of GH.

#### *Mutational screening of individuals with familial short stature*

Results presented herein show unaltered resistance of the Ile179Met GH variant to proteolytic cleavage compared to wild-type GH. This indicates that introduction of Met at position 179 does not cause significant misfolding of GH molecule. Molecular modelling did not indicate any significant structural defects associated with the Ile179Met variant.

#### *Proteolysis Studies*

Figure 1 shows the results of enzyme analysis performed on a number of GH variants in order to determine which, if any, of these variants alter the structural properties of the protein and so are likely to interfere with the activity thereof. 12 variants were examined and it can be seen that with respect to the wild-type, in the far left hand side of the Figure, the majority of these variants have an effect on the susceptibility of the protein to proteolytic digestion. The variants Thr27Ile

and Gln91Leu were particularly vulnerable to proteolysis and, in each case, proteolysis proceeded most efficiently using the enzyme chymotrypsin. In contrast, other variants showed only a marginal susceptibility to proteolysis such as VAL110Ile and Thr175Ala which were both most resistant to proteolysis by the enzyme chymotrypsin.

These results show that GH variants can be characterised in terms of their proteolysis signature in response to selected proteases and this information represents a first step towards selecting clinically and technologically important variants for further analysis.

**Table 1**

Auxological parameters and laboratory investigations for the patients with novel *GH1* mutations

Patient measurement	B49 (Ile179Met)	B4 (-360 A→G)	Group mean (SD) n=74
Chronological age (years)	6.9	6.0	8.6 (2.2)
Bone age (yrs)	6.4	6.6	8.0 (2.5)
Height (cm)	113	112.5	-
Height (SDS)	-2.7	-2.1	-2.4 (0.6)
Height velocity SDS	-1.6	-0.6	-1.1 (1.0)
Weight (kg)	19.0	17.8	-
Body mass index (SDS)	0.4	0.1	-0.5 (1.0)
Maternal height (cm)	138.9	148.7	-
Maternal height (SDS)	-3.9	-1.7	-
Paternal height (cm)	165.4	163.4	-
Paternal height (SDS)	-1.4	-2.3	-
Mid-parental height (SDS)	-2.4	-2.2	-
IGF-1 (SDS)	-1.2	-1.7	-0.9 (1.2)
IGFBP-3 (SDS)	1.5	0.4	0.1 (1.3)
GH peak (ng/ml)	10.4 (propanolol + exercise)	16.8 (clonidine)	17.6 (9.4)
GHBP (%)	27.5	29.8	27.8 (5.7)

\* All auxology data taken at this age.

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## CLAIMS

1. A method for determining the effect of a given nucleic acid polymorphism or mutation, in a nucleic acid molecule, on the structural properties of a protein encoded by said nucleic acid molecule comprising:
  - a) exposing the protein encoded by said nucleic acid molecule to at least one protease; and
  - b) determining whether or to what extent proteolytic cleavage takes place; and, optionally,
  - c) comparing this proteolytic cleavage with that of the wild-type protein when exposed to the same protease(s).
2. A method according to Claim 1 wherein said nucleic acid molecule is exposed to a plurality of proteases.
3. A method according to Claim 2 wherein at least some of said proteases are different and so attack different targets within the protein.
4. A method according to any preceding Claim wherein said protease(s) comprises any one or more of the following: trypsin, chymotrypsin proteinase K, aminopeptidase, carboxypeptidase, collagenase, elastase, Kallikrein, metalloendopeptidase, papain or pepsin.
5. A method according to any preceding Claim wherein a plurality of proteins



are exposed to said protease(s).

6. A method according to Claim 5 wherein said proteins are exposed to said protease(s) simultaneously.

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7. A method according to any preceding Claim wherein said protein(s) is exposed to said different proteases either simultaneously or successively.

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8. A method according to any preceding Claim wherein said protein(s) are exposed to said protease(s) under conditions that support the activity of said protease(s).

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9. A method according to any preceding Claim wherein digestion of said protein(s) is terminated by adding at least one protease inhibitor to the reaction.

10. A method according to any preceding Claim wherein proteolytic cleavage is determined using a conventional protein assay.

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11. A method according to Claim 10 wherein said assay involves SDS-PAGE analysis.

12. A method according to Claim 11 wherein said analysis is followed by staining or blotting.

13. A method according to any preceding Claim wherein additional studies are undertaken to determine the functionality of the protein variant.

5 14. A method according to any preceding Claim wherein part (a) involves further exposing the wild-type protein to said at least one protease and part (b) involves determining whether and to what extent proteolytic cleavage of said wild-type protein takes place.

10 15. A method according to any preceding Claim wherein the wild-type protein and, optionally, the variant protein are subjected to the conditions of the proteolytic reaction, in the absence of the said protease(s), and then the extent of proteolytic cleavage is determined.

## ABSTRACT

### PROTEOLYSIS METHODOLOGY

- 5      The invention relates to a method for determining the effect of a polymorphism or mutation on the structural properties of a protein wherein the method relies on the structural properties of the protein and so its cleavage during proteolysis.

# Enzyme Analysis of GH Variants

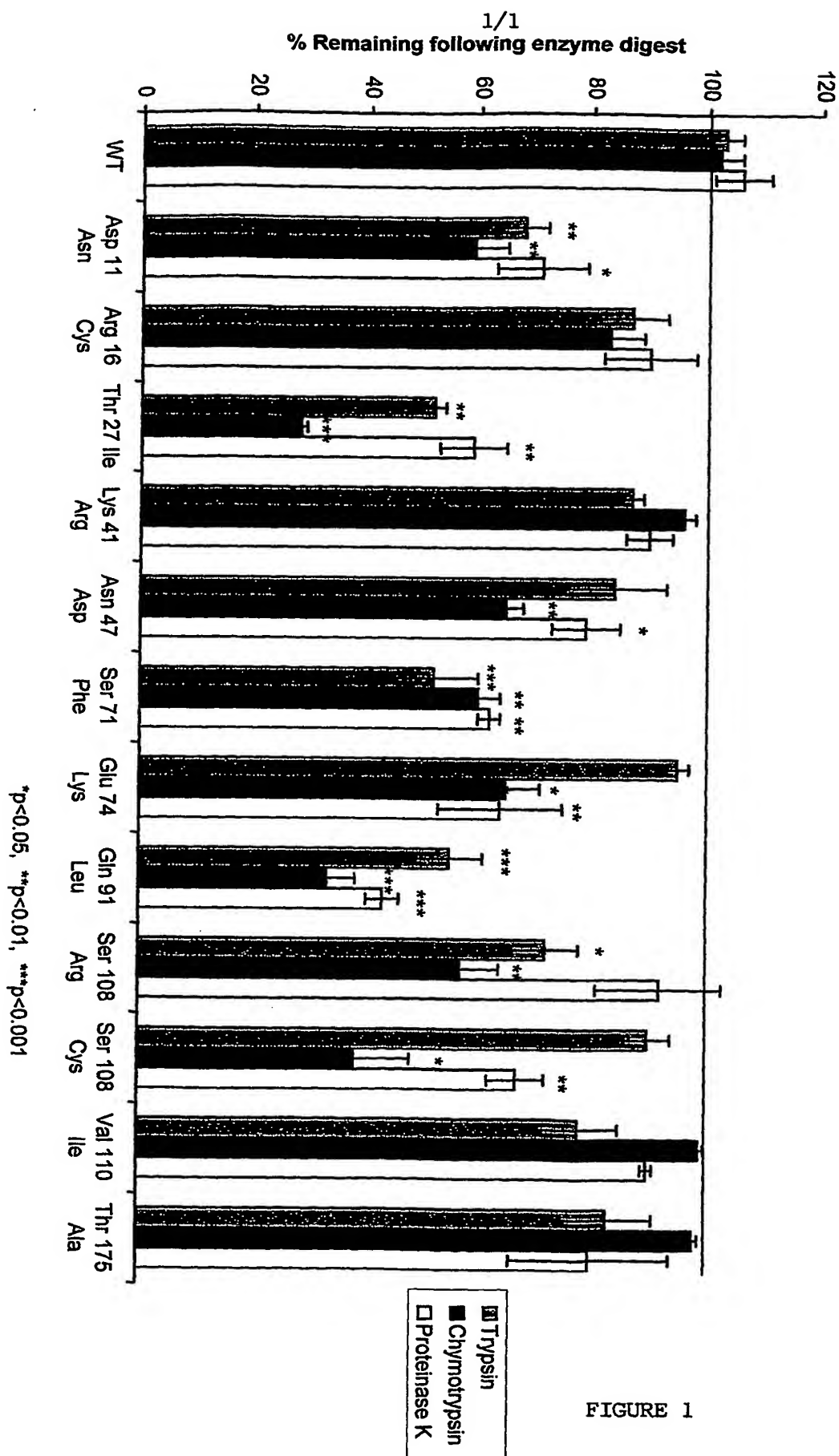


FIGURE 1

PCT Application

**GB0304740**



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